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ORIGINAL ARTICLE

Development and application of one-step multiplex reverse transcription PCR for simultaneous detection of five diarrheal viruses in adult cattle

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Abstract A one-step multiplex reverse transcription (RT)-PCR method was developed for the simultaneous detection of five viruses causing diarrhea in adult cattle: bovine group A rotavirus (GAR), bovine group B rotavirus (GBR), bovine group C rotavirus (GCR), bovine coronavirus (BCV), and bovine torovirus (BToV). The detection limit of the one-step multiplex RT-PCR for GAR, GCR, BCV, and BToV was 10^2, 10^0, 10^1, and 10^2 $TCID_{50}$/ml, respectively, and that for GBR was 10^6 copies/ml. The one-step multiplex RT-PCR with newly designed primers to detect GAR had higher sensitivity than a single RT-PCR with conventional primers, with no false-positive reactions observed for ten other kinds of bovine RNA viruses To assess its field applicability, 59 of 60 fecal samples containing one of these five viruses from all 25 epidemic diarrhea outbreaks in adult cattle were positive in the one-step multiplex RT-PCR assay. Furthermore, using four additional fecal samples containing two viruses (GBR and BCV or BToV), two amplified products of the expected sizes were obtained simultaneously. In contrast, all 80 fecal samples lacking the five target viruses from normal adult cattle were negative in the multiplex assay. Taken together, our results indicate that the one-step multiplex RT-PCR developed here for the detection of GAR, GBR, GCR, BCV, and BToV can be expected to be a useful tool for the rapid and cost-effective diagnosis and surveillance of viral diarrhea in adult cattle.

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Introduction

Epidemic outbreaks of diarrhea frequently occur in adult cattle, resulting in major economic losses to the dairy industry due to rapid decreases in milk production by affected cattle. Bovine coronavirus (BCV) is a primary cause of adult cattle diarrhea, including winter dysentery (WD), which is characterized by the sudden onset of epidemic diarrhea, which occasionally presents with bloody feces and reduced milk production during the winter season [15, 32, 36, 44]. In addition, bovine group A rotavirus (GAR) [14, 38], bovine group B rotavirus (GBR) [7, 18, 45], bovine group C rotavirus (GCR) [28, 42], and bovine torovirus (BToV) [19, 22] also have been identified in field cases of adult cattle diarrhea. However, the frequency of adult cattle diarrhea caused by these viruses remains unclear.

Rotaviruses are classified into seven groups (A-G) on the basis of antigenic and genomic analysis, and GAR, GBR, and GCR have been detected in cattle [12]. Bovine GAR is one of the major pathogens that causes acute diarrhea in neonatal calves worldwide [11] and is occasionally responsible for diarrhea in adult cattle [14, 38]. Bovine GBR and GCR have been associated with epidemic outbreaks of diarrhea in adult cattle but are relatively uncommon in calves [7, 18, 28, 42, 45], and in several outbreaks, marked drops in milk production have been observed [18, 28, 42]. Rotaviruses can be genotyped using the outer capsid proteins VP7 and VP4 to define the G and P genotype, respectively, based on sequence differences of the respective genes [12]. BToV, formerly called Breda virus, is a member of the genus *Torovirus* in the family *Coronaviridae* and was first detected from diarrheic calves in the United States in 1979 [47]. A few reports have found an association between BToV and adult cattle diarrhea, as well as calf diarrhea [19, 22].

Characterization of the clinical signs and epidemiology of these viral infections, with the exception of BCV, in adult cattle remains incomplete. Furthermore, as bloody feces are not always observed in BCV-associated diarrhea, viral detection is needed for differential diagnosis of viral diarrhea in adult cattle. To date, however, most diagnostic examinations have focused on BCV and GAR because commercial detection kits are only available for these two viruses [1, 14, 38]. As such, the isolation of GCR and BToV has only been reported in a few reports [26, 42], while GBR has not been successfully isolated to date [6, 46]. Reverse transcription PCR (RT-PCR) assays for sensitive and accurate detection of these RNA viruses has been used occasionally for the diagnosis of adult cattle diarrhea. However, conventional RT-PCR (single RT-PCR) assays targeting individual viruses is time-consuming, labor-intensive, and expensive. Thus, cost-effective, simple, and rapid laboratory assays are needed for the diagnosis of viral diarrhea in adult cattle.

Here, we attempted to develop a one-step multiplex RT-PCR assay for the simultaneous detection and differentiation of GAR, GBR, GCR, BCV, and BToV, and we evaluate the application of the assay using fecal samples collected from outbreaks of adult cattle diarrhea in Japan.

Materials and methods

Reference viruses

The bovine GAR strain Shimane (G6P[1]) [37] and nine bovine GAR field isolates from diarrheal adult cattle (one G8P[14], five G10P[11], one G6P[11], two GXP[11], and one G8P[X]; X indicates that the genotype could not be determined) were propagated in MA104 cells. The bovine GCR strain Shintoku [42] and BCV strains Kakegawa and Mebus [3, 29] were propagated in MA104 and HRT-18 cells, respectively. The BToV strain Aichi/2004 was propagated in HRT-18 cells [26]. The bovine GBR strain Nemuro was propagated in colostrum-deprived calves as described previously [45].

Ten other kinds of bovine RNA viruses were used as negative controls and propagated using standard techniques as follows: Bovine viral diarrhea virus (BVDV) type 1 strain Nose [24], BVDV type 2 strain KZ-91CP [31], bovine parainfluenza 3 virus strain BN-1 [20], and bovine rhinovirus strain M-17 [25] were propagated in MDBK cells. Akabane virus strain JaGAr-39 [34], Aino virus strain JaNAr-28 [41], Ibaraki virus strain Ibaraki [33], Chuzan virus strain K47 [30], and bovine ephemeral fever virus strain YHL [23] were propagated in HmLu-1 cells. Bovine respiratory syncytial virus strain NMK-7 [21] was propagated in Vero cells.

Fecal samples

A total of 60 diarrheal fecal samples collected from 60 adult cattle involved in 25 outbreaks of diarrhea between 2002 and 2011 in seven prefectures of Japan were used. The samples were positive for one of the five target viruses, as determined using conventional RT-PCR, as described below, a commercial antigen detection kit for GAR (Rotalex Dry, Orion Diagnostica, Espoo, Finland), or virus isolation [37]. Of the 60 fecal samples, eight were GAR positive from four outbreaks, eight were GBR positive from seven outbreaks, 10 were GCR positive from three outbreaks, 20 were BCV positive from eight outbreaks, and 14 were BToV positive from three outbreaks.

In addition, two fecal samples from calves infected with both GBR and BCV and two fecal samples from adult cattle infected with both GBR and BToV were also used. As negative controls, a total of 80 fecal samples collected in Saitama Prefecture, Japan, from clinically normal adult cattle that were negative for the five viruses by conventional RT-PCR assays were also used in the study.

To prepare samples for analysis, 10% fecal suspensions were made with Eagle's MEM (Nissui Pharmaceutical, Tokyo, Japan). After centrifugation of the suspensions for 6 min at 1,200×*g*, the resulting supernatant was stored frozen at −80 °C until use.

RNA extraction and single RT-PCR

RNA was extracted from 250 µl of virus-infected cell culture supernatants or fecal suspensions using ISOGEN-LS (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions.

Single RT-PCR was performed using previously reported primers for the detection of specific genes of GAR [16], GBR [9], GCR [43], BCV [44], and BToV [39] using a OneStep RT-PCR Kit (QIAGEN, Valencia, CA, USA).

Primers for multiplex RT-PCR

The sequences and characteristics of the five primer pairs used for one-step multiplex RT-PCR are shown in Table 1. The three primer pairs used for the detection of GBR, BCV, and BToV were the same as those used for single RT-PCR. A forward primer for GAR was previously reported [27]. A reverse primer for GAR detection and a primer pair for GCR detection were designed in this study.

Optimization of multiplex RT-PCR

Multiplex RT-PCR was performed using a OneStep RT-PCR Kit. To determine the optimum conditions for the assay, various primer concentrations (0.2-0.6 μM), annealing temperatures (45-60 °C), and numbers of amplification cycles (30-40 cycles) were examined using RNAs from all the reference viruses.

Sensitivity of single and multiplex RT-PCRs

The sensitivity of single and multiplex RT-PCR assays was evaluated on serial tenfold dilutions of RNAs of the target viruses. Prior to RNA extraction, GAR (Shimane), GCR (Shintoku), BCV (Mebus), and BToV (Aichi/2004) were adjusted to 10^5 median tissue culture infective dose ($TCID_{50}$) per ml with Eagle's MEM. RNAs were diluted serially with RNase-free water. For the sensitive assay against GBR, a plasmid containing the bovine GBR (Nemuro) VP7 gene was used. Briefly, a complete VP7 gene (816 bp) amplified by RT-PCR [45] was ligated into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA), and mRNA was then generated from the plasmid DNA using SP6 RNA polymerase (Roche Applied Science, Penzberg, Germany) and purified by ethanol precipitation. Tenfold serial dilutions of the mRNA were prepared from a starting solution containing 10^9 copies per ml using RNase-free water. The limit of detection was reported as $TCID_{50}$ or RNA copies per ml.

Results

Optimization of the one-step multiplex RT-PCR

The optimal conditions for the one-step multiplex RT-PCR included the use of a 0.4 μM concentration of each primer, an annealing temperature of 52 °C, and 35 amplification cycles. For the assay, 2 μl of extracted RNA was first heated with 1 μl of dimethyl sulfoxide and 12.2 μl of RNase-free water at 98 °C for 5 min, and the temperature was then held at 4 °C. A reaction mixture consisting of 5 μl 5× buffer, 1 μl dNTP Mix (10 mM each dNTP), and 1 μl enzyme mix from the OneStep RT-PCR Kit, 0.3 μl RNase OUT (Invitrogen), and 2.5 μl 10x primer mix (4 μM each primer) was then added to the RNA solution. The RT-PCR reaction was performed at 50 °C for 30 min and 95 °C for 15 min, followed by 35 cycles of 94 °C

Table 1 Primers used in the one-step multiplex RT-PCR

Virus	Target gene	Primer	Sequence (5′–3′)[a]	Position	Product length (bp)	Reference No.
GAR	VP6	GEN_VP6F	GGCTTTWAAACGAAGTCTTC	1–20[b]	928	[27]
		GAR VP6-928R	GGYGTCATATTYGGTGG	928–912[b]		Newly designed
GBR	VP7	9B3	CAGTAACTCTATCCTTTTACC	171–191[c]	281	[9]
		9B4	CGTATCGCAATACAATCCG	451–433[c]		
GCR	VP6	ShintokuVP6-370F	ATCGCATTAGCTTCATCAAA	370–389[d]	563	Newly designed
		ShintokuVP6-933R	CTGTACATACTGGGTCATAGC	933–913[d]		
BCV	Nucleocapsid	BCV-N-F	GCCGATCAGTCCGACCAATC	29475–29494[e]	407	[44]
		BCV-N-R	AGAATGTCAGCCGGGGTAT	29881–29863[e]		
BToV	Nucleocapsid	1344	GAGAAAGAGCCAAGATGAATT	27761–27781[f]	664	[39]
		294	CTTACATGGAGACACTCAACCA	28424–28403[f]		

[a] W = A or T; Y = C or T

[b] Based on the VP6 gene of strain NCDV (accession no. AF317127)

[c] Based on the VP7 gene of strain Nemuro (accession no. AB016818)

[d] Based on the VP6 gene of strain Shintoku (accession no. M88768)

[e] Based on the complete genome of strain Mebus (accession no. U00735)

[f] Based on the complete genome of strain Breda 1 (accession no. AY427798)

for 45 sec, 52 °C for 45 sec, and 72 °C for 1 min, and incubation at 72 °C for 10 min, after which the temperature was held at 4 °C. Under optimal conditions, only products of the expected sizes were amplified from RNA of the reference viruses, and these could be easily distinguished from each other following agarose gel electrophoresis (Fig. 1a). At annealing temperatures below 50 °C, nonspecific amplification products frequently appeared, while at temperatures above 54 °C, the amount of specific product for GAR, GBR, and BToV was clearly lower than that obtained at 52 °C (data not shown).

Sensitivity comparison of single and one-step multiplex RT-PCRs

As shown in Fig. 2, the detection limits of the single and multiplex RT-PCRs were 10^4 and 10^2 $TCID_{50}$/ml, respectively, for GAR, 10^1 and 10^2 $TCID_{50}$/ml for BToV, 10^1 and 10^0 $TCID_{50}$/ml for GCR, 10^2 and 10^1 $TCID_{50}$/ml for BCV, and 10^5 and 10^6 copies/ml for GBR.

Specificity testing of one-step multiplex RT-PCR

In the one-step multiplex RT-PCR for the five target viruses, no detectable amplification occurred with samples of BVDV type 1, BVDV type 2, bovine parainfluenza 3 virus, bovine rhinovirus, bovine respiratory syncytial virus, Akabane virus, Aino virus, Ibaraki virus, Chuzan virus, bovine ephemeral fever virus, or bovine respiratory syncytial virus. Furthermore, no PCR products were observed in assays performed with 80 fecal samples that were negative for target viruses. By contrast, all target viral genes were specifically amplified by the assay using the reference viruses (Fig. 1b, lane 6).

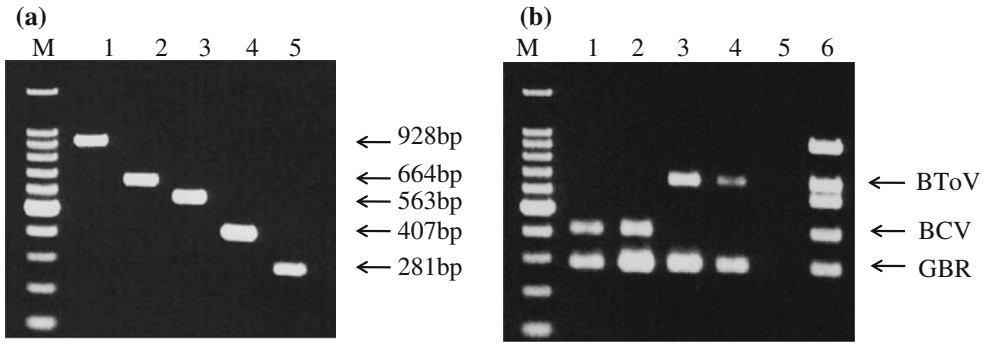

Fig. 1 One-step multiplex RT-PCR products generated under optimal conditions for five reference viruses (**a**) and for fecal samples collected from cattle with concurrent infection caused by GBR and BCV or BToV (**b**). (**a**) Lane 1, GAR (strain Shimane); lane 2, GBR (strain Nemuro); lane 3, GCR (strain Shintoku); lane 4, BCV (strain Mebus); lane 5, BToV (strain Aichi/2004); lane M, 100-bp DNA ladder marker. (**b**) Lanes 1 and 2, fecal samples from GBR- and BCV-infected calves; lanes 3 and 4, fecal samples from GBR- and BToV-infected adult cattle; lane 5, negative control (water); lane 6, positive control (mixture of reference viral RNAs); lane M, 100-bp DNA ladder marker

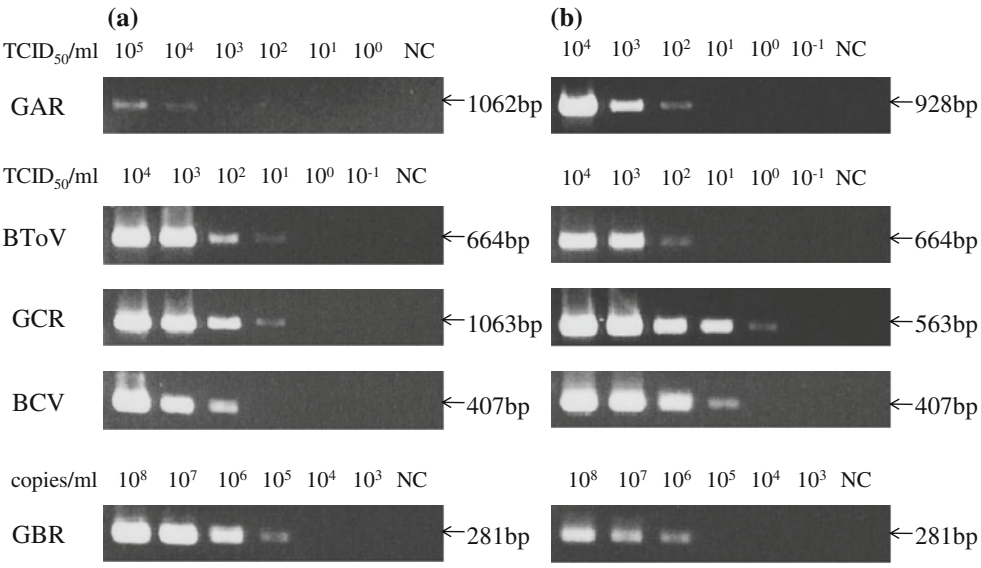

Fig. 2 Sensitivity of conventional single RT-PCR (**a**) and one-step multiplex RT-PCR (**b**) for GAR (strain Shimane), BToV (Aichi/2004), GCR (strain Shintoku), BCV (Mebus), and GBR (Nemuro). Reactions were performed using tenfold serial dilutions of viral RNA. NC, negative control

Table 2 Results of the single and one-step multiplex RT-PCRs[a] in 60 virus-positive fecal samples at 25 outbreaks

Virus	Outbreak no.	Sample no.	Single RT-PCR	Multiplex RT-PCR
GAR	1	1	+	+
		2	+	+
	2	3[b]	−	+
		4	+	+
	3	5	+	+
		6	+	+
	4	7[b]	−	+
		8[b]	−	+
GBR	5	9	+	+
	6	10	+	+
	7	11	+	+
	8	12	+	+
	9	13	+	+
	10	14	+	+
	11	15[c]	+	−
		16	+	+
GCR	12	17	+	+
		18	+	+
		19	+	+
	13	20	+	+
		21	+	+
		22	+	+
	14	23	+	+
		24	+	+
		25	+	+
		26	+	+
BCV	15	27	+	+
		28	+	+
	16	29	+	+
		30	+	+
	17	31	+	+
		32	+	+
	18	33	+	+
		34	+	+
	19	35	+	+
		36	+	+
	20	37	+	+
		38	+	+
	21	39	+	+
		40	+	+
	22	41	+	+
		42	+	+
		43	+	+
		44	+	+
		45	+	+
		46	+	+
BToV	23	47	+	+
		48	+	+
		49	+	+
		50	+	+
		51	+	+
		52	+	+
	24	53	+	+
		54	+	+
		55	+	+
		56	+	+
	25	57	+	+
		58	+	+
		59	+	+
		60	+	+

[a] Positive and negative reactions are + and −, respectively

[b] GAR was detected by Rotalex Dry Kit and virus isolation

[c] GBR was confirmed by direct sequencing

Application of the one-step multiplex RT-PCR with virus-positive field samples

The results of the single and one-step multiplex RT-PCRs for 60 virus-positive fecal samples collected during 25 outbreaks are summarized in Table 2. Consistent results were obtained for both assays, with the exception of outbreaks 2, 4 and 11. For outbreaks 2 and 4, the single RT-PCR failed to detect GAR in one or two samples, whereas the one-step multiplex RT-PCR detected GAR in these samples (Table 2, no. 3, 7 and 8). The presence of GAR in these samples had been confirmed using a Rotalex Dry kit and virus isolation. For outbreak 11, the one-step multiplex RT-PCR failed to detect GBR in one sample that was positive in the single RT-PCR (Table 2, no. 15). The presence of GBR in the sample was confirmed by direct sequencing of the single RT-PCR product (data not shown). No false-positive reactions were observed in any sample for either assay. When additional fecal samples with concurrent infections with GBR, and BCV or BToV were used in the one-step multiplex RT-PCR, two amplified products of the expected sizes were detected simultaneously (Fig. 1b, lanes 1-4).

Discussion

In newborn calves, GAR and BCV are common causes of viral diarrhea [1]; however, recent reports indicate that in addition to these viruses, GBR, GCR, and BToV are also associated with epidemic diarrhea in adult cattle. Although

the simultaneous detection of GAR and BCV by multiplex RT-PCR assays has been reported [4, 10], to our knowledge, this represents the first report of a multiplex RT-PCR for the simultaneous detection of all five viruses.

In most previous studies on multiplex RT-PCR for detecting diarrheal viruses in calves, pigs, and humans, two-step assays in which the RT and PCR reactions are performed separately were adopted [4, 5, 17, 35, 40]. Therefore, here, we initially assessed a two-step multiplex RT-PCR assay using random primers in the RT step. However, the one-step multiplex RT-PCR assay showed greater sensitivity and specificity than the two-step assay (data not shown). Furthermore, as one-step assays are simpler and more convenient than two-step assays, the practical application of the one-step multiplex RT-PCR method developed in this study was further evaluated.

Diarrhea caused by BVDV infection is a symptom of mucosal disease in persistently infected cattle, or of acute infection in seronegative immunocompetent cattle [13]. However, BVDV-associated diarrhea occurs sporadically and is primarily observed in cattle under 24 months of age [13]. As mucosal disease is systemic and fatal, it is not difficult to make a clinical diagnosis. Furthermore, the incidence of diarrhea resulting from acute BVDV infection in adult cattle is extremely low based on our recent epidemiological survey (manuscript in preparation). Therefore, in this study, we did not include BVDV as a target virus for detection.

When the sensitivity was compared between the single and multiplex RT-PCRs against each of the five target viruses, a relatively large difference was observed for the detection of GAR. Notably, the sensitivity of the multiplex RT-PCR against GAR strain Shimane RNA was approximately 100-fold higher than that of the single RT-PCR. A similar difference was observed when the assays were used to assess GAR-positive fecal samples. Although the reason for the markedly reduced sensitivity of the single RT-PCR remains unclear, one possibility is that the primers (Beg9 and End9) [16] used for the assay might be unsuitable for the detection of some GAR strains.

A significant finding of our study was that the one-step multiplex RT-PCR could detect concurrent infections with different viruses for both laboratory and clinical samples. Chang et al. [8] examined concurrent infections with GAR and GCR in diarrheic adult cattle and found that dual infection with these two viruses in gnotobiotic calves enhanced the pathogenesis of both viruses. However, the diagnosis of adult cattle diarrhea is typically conducted against only BCV and GAR. Because BCV is a cause of WD, GAR can be easily examined with rapid diagnostic kits, such as immune chromatography and latex agglutination tests [2, 14, 38]. The routine use of the one-step multiplex RT-PCR assay developed here may allow elucidation of the epidemiology of these viral infections and detection of concurrent infections in adult cattle.

In conclusion, we have developed a one-step multiplex RT-PCR assay that is capable of detecting five viruses causing acute diarrhea in adult cattle. The sensitivity of the assay was similar to that of the single RT-PCR technique for all of the reference viruses examined, with the exception of GAR. Notably, the one-step multiplex RT-PCR was able to detect the causative agents for all 25 outbreaks of epidemic diarrhea that were examined. In addition, the required amounts of samples and reagents and inspection times can be decreased using this assay. Taken together, the one-step multiplex RT-PCR assay developed in this study may be a useful tool for the rapid and cost-effective diagnosis of the causative agents of viral diarrhea in adult cattle.

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